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# Glycoprotein gIII of Pseudorabies Virus Is Multifunctional

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**One of the major glycoproteins of pseudorabies virus, gIII, is nonessential for growth in cell culture. Mutants defective in gIII, however, consistently yield lower titers of infectious virus (3- to 20-fold) than does wild-type virus. The interactions of gIII$^-$ mutants with their host cells were compared with those of wild-type virus in an attempt to uncover the functions of gIII. We show that gIII plays a major role in the stable adsorption of the virus to its host cell; in the absence of gIII, the rate of adsorption is reduced and adsorption is easily reversed by washing. Thus, adsorption of pseudorabies virus can be said to occur in at least the following two ways: (i) a gIII-mediated rapid adsorption or (ii) a slower and more labile adsorption that is independent of gIII. After virions have been complexed with monoclonal antibodies against gIII (but not some monoclonal antibodies against other glycoproteins), both modes of adsorption were inhibited. Glycoprotein gIII affects virus stability and virus release, as well as adsorption. The effect on virus release is marked when the virus is defective in additional functions. Thus, although we found no obvious difference in the release of virus from gIII$^-$ or wild-type virus-infected rabbit kidney cells, release of a gIII$^-$/gI$^-$ double mutant from the cells occurred less readily than did release of a gI$^-$ mutant. The gIII$^-$/gI$^-$ and gIII$^-$ mutants, however, adsorbed to cells at a similar rate, indicating that the effects of gIII on adsorption and virus release constitute separate functions. The Bartha vaccine strain of pseudorabies virus has a defective gIII gene and is released poorly from rabbit kidney cells. After the resident Bartha gIII gene was replaced by the gIII gene of wild-type virus, virus release was enhanced considerably. Since inactivation of gIII in wild-type pseudorabies virus did not significantly affect virus release, the Bartha strain must be defective in another function which, in conjunction with gIII, significantly affects virus release. These results indicate again that gIII affects virus release in conjunction with other functions. Also, although the Bartha strain was functionally defective in virus release, it adsorbed to cells as well as wild-type virus did, showing that the effects of gIII on virus adsorption and release constitute separate functions. We conclude that gIII is a multifunctional glycoprotein.**

Pseudorabies virus (PrV), a herpesvirus of swine, encodes at least eight different glycoproteins. Six of the genes encoding these glycoproteins have been mapped and sequenced (18, 20–22, 24, 26, 27, 31); four of the glycoproteins are nonessential for growth in cell culture (1, 17, 21, 28, 32).

We have been interested in ascertaining the functions of the glycoproteins of PrV in virulence, in immunity, and in the interactions of the virus with cells in culture. To this end, we constructed mutants defective in one or more nonessential glycoproteins and initiated studies in which their role in the infective process and in virulence was ascertained.

One of the major nonessential glycoproteins of PrV is gIII, a homolog of gC of herpes simplex virus (HSV) (27). Glycoprotein gIII is not necessary for growth of PrV in vitro (1, 28, 32), but strains that produce no gIII (or a truncated gIII) give rise to lower titers of infectious virus than does the wild type (11, 29). These studies were designed to clarify the role of gIII in the infective process.

We report here that cells infected with gIII$^-$ mutants of PrV produce virion populations that give rise to a lower titer of infectious virus than does the wild type, confirming previous findings (11, 29). The lower titer of gIII$^-$ mutants can be attributed to the fact that gIII plays a major role in the stable adsorption of the virus to its host cells; this observation is consistent with our previous findings that monoclonal antibodies against gIII interfere with virus infectivity, even in the absence of complement (7). Furthermore, we show that gIII also affects virus stability and plays a role in virus release. The latter effect was, however, significant and easily detectable in rabbit kidney cells only in conjunction with defects in other viral functions; i.e., it is dependent upon the genetic background of the virus strain.

We conclude from our results that gIII, although not essential for growth in cell culture, is nevertheless important to several processes involved in the interactions of the virus with its host cells and that gIII is a multifunctional glycoprotein.

## MATERIALS AND METHODS

**Virus strains and mutants and cell cultures.** Rabbit kidney (RK) cells, pig kidney (PK) cells, and MDBK cells were cultivated in Eagle synthetic medium containing 3% dialyzed bovine serum; the virus was titrated by plaque assay in RK or MDBK cells. PrV(Ka) is a strain which has been carried in our laboratory for more than 25 years; its origin is uncertain (10). Mutants of PrV(Ka) defective in the expression of gIII were constructed essentially by the methods of Robbins et al. (27), as described in greater detail elsewhere (T. C. Mettenleiter, C. Schreurs, F. Zuckermann, T. Ben-Porat, and A. S. Kaplan, J. Virol., in press). Deletions in the gIII gene were introduced into wild-type PrV(Ka) or into PrV(Ka) mutants which had a deletion in the gene encoding glycoprotein gI, PrV(Ka)Δ1 (19). The monoclonal antibody-resistant (MAR) mutant of PrV(Ka), M7p1, defective in the expression of gIII, was isolated by the method of Holland et al. (8); its characteristics have been described previously (1).

The Bartha avirulent vaccine strain was provided by P. S.

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TABLE 1. Immunoprecipitation of glycoproteins of various variants of the Bartha strains[a]

| Virus | Amt immunoprecipitated ($10^3$ cpm) with indicated monoclonal antibody: | | | | | | |
|---|---|---|---|---|---|---|---|
| | gII (M2) | gI | | gp63 (3M8) | gIII | | |
| | | M133 | M156 | | M1 | M7 | M16 |
| PrV(Ka) mutants | | | | | | | |
| PrV(Ka) (wild type) | 225 | 123 | 146 | 79 | 298 | 208 | 306 |
| PrV(Ka)gIII$^-$ | 275 | 113 | 173 | 59 | 0 | 0 | 0 |
| PrV(Ka)gIII$^-$/gI$^-$ | 295 | 0 | 0 | 49 | 0 | 0 | 0 |
| MARgIII$^-$ (M7P1) | 305 | 133 | 117 | ND[b] | 0 | 0 | 0 |
| Bartha variants | | | | | | | |
| Parental Bartha | 257 | 0 | 0 | 0 | 110 | 110 | 0 |
| Bartha gIII$^{Ka}$ | 217 | 0 | 0 | 0 | 259 | 259 | 257 |
| Bartha 43/25a | 182 | 93 | 113 | 37 | 167 | 167 | 0 |
| Bartha 43/25a/gIII$^{Ka}$ | 219 | 81 | 83 | 42 | 315 | 315 | 301 |

[a] [$^{35}$S]methionine-labeled cell extracts obtained from RK cultures infected with the indicated virus variants were immunoprecipitated with the indicated monoclonal antibodies. The amount of radioactivity immunoprecipitated was determined. The immunoprecipitated glycoproteins were identified by polyacrylamide gel electrophoresis (data not shown).

[b] ND, Not done.

TABLE 2. Replication of wild-type virus PrV(Ka) and gIII mutants in cell culture[a]

| Time after infection (h) | Amt (PFU/ml) of: | | |
|---|---|---|---|
| | PrV(Ka) | PrV(Ka)gIII$^-$ | M7p1 |
| Expt 1 | | | |
| 2.5 | $3.2 \times 10^4$ | $2.2 \times 10^2$ | $2.1 \times 10^2$ |
| 24.0 | $3.5 \times 10^7$ | $4.9 \times 10^6$ | $3.2 \times 10^6$ |
| 48.0 | $4.2 \times 10^7$ | $1.0 \times 10^7$ | $7.2 \times 10^6$ |
| Expt 2 | | | |
| 3.0 | $2.1 \times 10^4$ | $4.5 \times 10^1$ | $3.9 \times 10^2$ |
| 28.0 | $9.0 \times 10^7$ | $3.0 \times 10^6$ | $1.0 \times 10^7$ |
| 48.0 | $1.9 \times 10^8$ | $1.9 \times 10^7$ | $3.8 \times 10^7$ |

[a] Primary RK cells were grown to confluence in 90-mm petri dishes and infected with virus (4 PFU per cell). After 1 h of adsorption, the cell monolayers were washed extensively to remove unadsorbed virus and further incubated at 37°C in 10 ml of medium. At the indicated times, cells were scraped into the culture field, the samples were sonicated for 1 min and centrifuged at 7,000 × *g* for 10 min to remove cellular debris, and the virus was plaque assayed on RK cells.

Paul. The origin of this strain has been described previously (23). The Bartha strain has a deletion in the S component, which includes the genes encoding the glycoproteins gp63 and gI (14, 17, 21). Bartha 43/25a is a strain to which an intact S component has been restored and which expresses gI and gp63 (15).

The Bartha vaccine strain and its derivatives encode a gIII glycoprotein (gIII$^B$) that differs from that of wild-type PrV(Ka) (gIII$^{Ka}$) in the following two respects. (i) At most, only 50% as much glycoprotein gIII accumulates in cells infected with the Bartha strain as with wild-type PrV(Ka). (ii) Only 10% as much gIII is present in Bartha virions as in PrV(Ka) virions (1, 3; F. Zuckermann and T. Ben-Porat, unpublished results). These characteristics are encoded in the gene encoding gIII, since transfer of the Bartha gIII gene to a gIII$^-$ wild-type virus resulted in the expression of a Bartha-like gIII (L. Enquist, personal communication). Furthermore, replacement of the Bartha gIII gene with a PrV(Ka) gIII gene resulted in the expression of a PrV(Ka)-like gIII (see below). The resident gIII$^B$ gene in the Bartha strain and its variant was replaced by the gIII$^{Ka}$ gene of PrV(Ka) by marker rescue, as follows. Cells were cotransfected either with parental Bartha DNA or with DNA of the marker-rescued Bartha variant and the *Pst*I 4.3-kilobase fragment of PrV(Ka) (which includes the regulatory and coding sequences of gIII [27]). The virus progeny was plaque assayed, and the plaques were stained with immunoperoxidase (12) by using a monoclonal antibody against gIII (M16) that reacts with the PrV(Ka) glycoprotein gIII (gIII$^{Ka}$) but that does not react with the Bartha glycoprotein gIII (gIII$^B$). Plaques that reacted with monoclonal antibody M16 were picked, replaque purified, and restained to confirm their gIII$^{Ka}$ genotype. Their identity as Bartha variants was established by their characteristic restriction enzyme profiles (data not shown).

The reactivities of the proteins synthesized by cells infected with the various virus strains and mutants with monoclonal antibodies against glycoproteins gI, gII, gp63, or gIII are summarized in Table 1. Some of these data will be published elsewhere (Mettenleiter et al., in press) and are presented here to prove the identity of the phenotype of the mutants used in this study.

**Immunoprecipitation.** The monoclonal antibodies against gI, gII, and gIII have been described previously (1, 3, 8). The monoclonal antibodies against gp63 (3M8) and gVI (gp50) were generous gifts of P. Desmettre, Rhone-Merieux, and Carmine Marchioli, The Upjohn Co., respectively. Immunoprecipitation was performed as described previously (8).

**Purification of virions.** Extracellular and intracellular viruses were purified as described previously (2).

## RESULTS

**Growth of gIII$^-$ mutants in cell culture.** The ability of the various mutants to grow in RK cells was determined. Table 2 shows the results of two one-step growth curve experiments performed with wild-type PrV(Ka), a gIII$^-$ deletion mutant of PrV(Ka), as well as a MAR mutant defective in the synthesis of gIII, M7p1. Two observations are noteworthy. First, the final titer of infectious virus obtained with the gIII$^-$ mutants was somewhat lower than that obtained with wild-type virus. In these particular experiments it was between 5 and 10 times lower. In other experiments the difference might be as high as 20-fold or as low as 3-fold. The lower titer of infectious virus obtained from gIII$^-$-infected cells was reproducibly obtained; these observations confirm previous reports by Enquist and co-workers (11, 29). Second, although the cultures were infected in each case with 4 PFU per cell and were washed similarly to remove the unadsorbed inoculum, the titer of virus obtained during the eclipse phase (2.5 to 3 h postinfection) was much higher (approximately by 100-fold) in the wild-type virus-infected cells than in gIII$^-$ mutant-infected cells.

Several possibilities may explain the smaller amount of infectious virus obtained from gIII$^-$ mutant-infected cells than from wild-type-infected cells. (i) The gIII$^-$ mutants do not adsorb as well to the host cells as does wild-type virus. This would explain the low titer obtained during the eclipse phase (Table 2) with the gIII$^-$ mutant-infected cells. (ii) The gIII$^-$ mutants are less stable than are wild-type virions. (iii) The gIII$^-$ mutants are released less efficiently from the cells than are wild-type virions. (iv) Cells infected with gIII$^-$ mutants produce fewer virus particles than do cells infected with wild-type virus. The experiments described below were designed to distinguish among these possibilities.

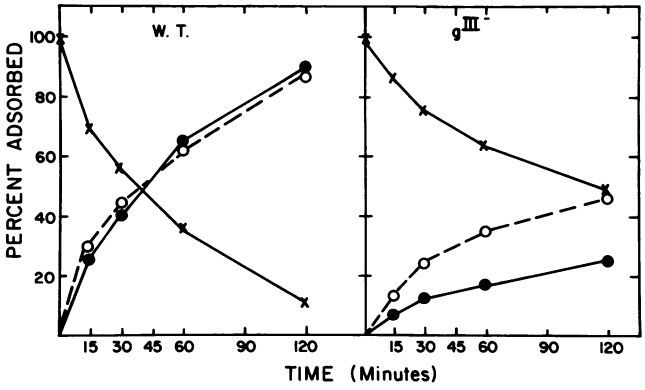


FIG. 1. Adsorption of wild-type (W.T.) virus PrV(Ka) and a gIII⁻ mutant to MDBK cells. The virus preparations were diluted in medium to give approximately 150 PFU/ml, and 1 ml of each was added to cultures of MDBK cells grown in 50-mm petri plates. At the indicated time intervals, the inocula were removed and the plates were overlaid with agarose either without being washed (○) or after being washed (●). The inocula that had been removed were added to fresh plates that were further incubated for 1 h before agarose was added (×). Plaques were counted after 4 days of incubation.

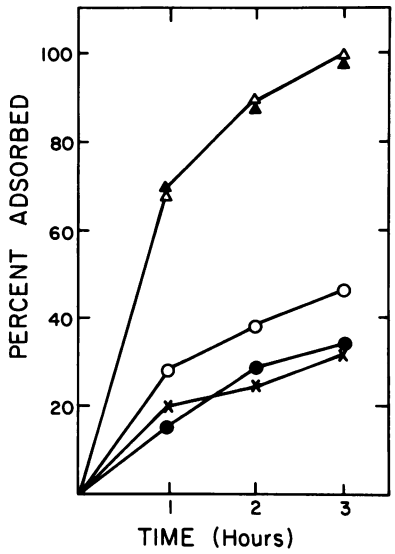


FIG. 2. Adsorption of various mutants of PrV(Ka) to MDBK cells. The virus preparations were diluted in medium to give approximately 100 PFU/ml, and 1 ml of each was added to cultures of MDBK cells. At the indicated times, some of the cultures were washed extensively and overlaid with agarose. Plaques were counted after 4 days of incubation. Symbols: ▲, wild-type PrV(Ka); △, PrV(Ka)gI⁻ deletion mutant: ○, PrV(Ka)-MAR gIII⁻ (M7p1); ●, PrV(Ka)gIII⁻ deletion mutant; ×, PrV(Ka)gIII⁻/gI⁻ deletion mutant.

**Effect of gIII on virus adsorption.** Figure 1 shows the results of a representative experiment in which the adsorption to MDBK cells of wild-type PrV(Ka) and of a gIII⁻ deletion mutant was tested. The gIII⁻ deletion mutant did not adsorb as rapidly to MDBK cells as did wild-type virus, as determined by the amount of infectious virus remaining in the adsorption fluid; by 2 h postinoculation, approximately 90% of the wild-type virus, but only 50% of the gIII⁻ mutant, had disappeared from the adsorption fluid. In both cases, the infectious virus that had disappeared from the adsorption fluid was, as expected, associated with the cell monolayer, provided that the cell monolayer was not washed. However, whereas the wild-type virus that was associated with the cell monolayer could not be removed by washing, a large proportion of the gIII⁻ mutants that had "adsorbed" to the cell surface were removed from the monolayer by washing.

That the slow and unstable adsorption to host cells of the gIII deletion mutant used in this experiment was not confined to that particular mutant is shown by the results summarized in Fig. 2. In this experiment, two independently isolated gIII⁻ deletion mutants, gIII⁻ and gIII⁻/gI⁻, as well as a MAR mutant (M7p1) that fails to express any detectable amount of gIII (1), also did not adsorb as well to MDBK cells as did PrV(Ka) wild-type virus or a gI⁻ deletion mutant of PrV(Ka). These results indicate that gIII⁻ mutants are deficient in their ability to adsorb in a stable manner to cells, i.e., that gIII plays a role in virus adsorption. However, since gIII⁻ mutants do produce plaques (i.e., are infectious), it is clear that they can adsorb to cells and can initiate infection (although at a much lower rate). Therefore, a mode of adsorption of this virus to the cells, independent of gIII, must exist.

Figures 1 and 2 show a comparison of the percentage of plaques formed when the inoculum was removed from the cells after the adsorption period and the percentage formed when it was not removed before the cultures were overlaid with agarose. The slow adsorption of the gIII⁻ mutants must therefore have continued even after the agarose had been added to the monolayer. Because adsorption of the virus to the cells is probably inefficient after the cultures have been overlaid with agarose, the lower titer of infectious virus obtained from gIII⁻-infected cells than from wild-type-infected cells (Table 2) may be the result of poor adsorption of the gIII⁻ mutants. Therefore, we performed an experiment to test whether the difference in the number of plaques obtained from wild-type and gIII⁻ virus preparations might vary with the conditions of adsorption used during virus titration.

The concentration of the virus in the inoculum affects the titer of gIII⁻ mutants to a greater extent than it affects the titer of wild-type PrV (Table 3). In this particular experiment, the difference between the number of PFU obtained with the preparations of wild-type virus and of gIII⁻ virus decreased from approximately fivefold to a little more than twofold, depending on the volume of the inoculum used. It is likely, therefore, that the difference in the number of PFU obtained from cells infected with gIII⁻ mutants and wild-type virus is due, at least in part, to the inefficient adsorption of the former. Thus, it appears that the estimation of the number of PFU in a preparation of gIII⁻ mutants is inexact and will depend on the conditions of the assay.

TABLE 3. Effect of the volume of virus inoculum on the virus titer of the wild type and gIII⁻ mutants[a]

| Virus | Virus titer in PFU/ml (%) in following adsorption vol: | |
|---|---|---|
| | 1 ml | 0.2 ml |
| PrV(Ka) wild type | $1.1 \times 10^8$ (100) | $2.6 \times 10^8$ (100) |
| PrV(Ka)gIII⁻ | $2.3 \times 10^7$ (21) | $1.2 \times 10^8$ (46) |

[a] Cultures of MDBK cells grown in 50-mm plastic dishes were infected with either 1 or 0.2 ml of 10-fold dilutions of the virus stocks. After 1 h of adsorption, the cultures were overlaid with agarose and incubated for 4 days at 37°C in a $CO_2$ incubator, and the number of plaques was counted.

TABLE 4. Effect of different monoclonal antibodies on the infectivity of PrV[a]

| Monoclonal antibody | Mean no. of plaques from[b]: | |
|---|---|---|
| | Washed culture | Unwashed culture |
| None (control) | 72 | 88 |
| M1 (gIII) | 0 | 0 |
| M16 (gIII) | 2 | 10 |
| M7 (gIII) | 0 | 6 |
| M6 (gIII) | 0 | 6 |
| M2 (gII) | 66 | 81 |
| M138 (gI) | 58 | 68 |
| M156 (gI) | 72 | 72 |
| 3M8 (gp63) | 62 | 68 |
| anti-gp50 | 54 | 48 |

[a] PrV(Ka) was diluted to approximately 80 PFU/ml and incubated with the indicated monoclonal antibody (final dilution, 1:100) for 1 h at 37°C. Cultures of MDBK cells in 50-mm plastic dishes were infected with 1 ml of virus and incubated for 2 h at 37°C. Part of the cultures was then extensively washed to remove the nonadsorbed inoculum; part of the cultures was not washed, and the inoculum was not removed. The cultures were overlaid with agarose and incubated at 37°C for 4 days.

[b] Mean value from triplicate cultures.

**Effect of monoclonal antibodies against gIII on virus adsorption.** The results described above indicate that although adsorption of PrV to the cells is enhanced by gIII, it is not absolutely dependent on it. Thus, initiation of infection can occur by (i) a rapid mode of gIII-dependent adsorption of the virus to the cell or (ii) a slower, gIII-independent association of the virus with the cells. It is possible that the two modes of adsorption of the virus to the cells occur by different mechanisms. Alternatively, gIII may enhance the process of virus adsorption to the cells.

We have shown previously that monoclonal antibodies against gIII inhibit adsorption of the virions to RK cells. In these experiments, the inoculum had been removed from the cultures after an adsorption period of 60 min. Therefore, the effect of the monoclonal antibodies on adsorption could have been restricted to the gIII-dependent rapid adsorption. The experiments below were performed to determine whether, indeed, monoclonal antibodies against gIII inhibit rapid gIII-dependent adsorption only or whether they also interfere with the slower mode of adsorption.

In the absence of complement, all the monoclonal antibodies against gIII used in this experiment inhibited the formation of plaques (Table 4). This was true whether or not the inoculum was removed after a 2-h adsorption period. While some plaques developed on the plates inoculated with virus treated with monoclonal antibodies against gIII, even though the inoculum was allowed to remain when the agarose was added, they constituted only a small percentage of the initial inoculum. Thus, virus which has become complexed with monoclonal antibodies against gIII is unable to initiate infection, whereas virus that is deficient in gIII can initiate infection, but does so much more slowly. It appears, therefore, that the gIII-independent mode of adsorption does not function when the virus is complexed with antibodies against gIII. Monoclonal antibodies against gIII therefore interfere not only with the primary (gIII-mediated) adsorption, but also with the slower (gIII-independent) mechanism of adsorption to the cells. On the other hand, several monoclonal antibodies against gI and gII, as well as one each against gp63 and gp50, which also complex with the virions (8; T. Ben-Porat, unpublished results), interfered only slightly (if at all) with adsorption.

**Effect of mutations in gIII on virus stability.** Although the results described above clearly implicate gIII in virus adsorption, an experiment was performed to ascertain whether this glycoprotein also plays a role in other aspects of the biology of the virus. A mutant with a deletion in the gIII gene was more thermolabile than was wild-type virus (Fig. 3). Thus, in addition to being involved in virus adsorption, gIII also plays a role in virus stability.

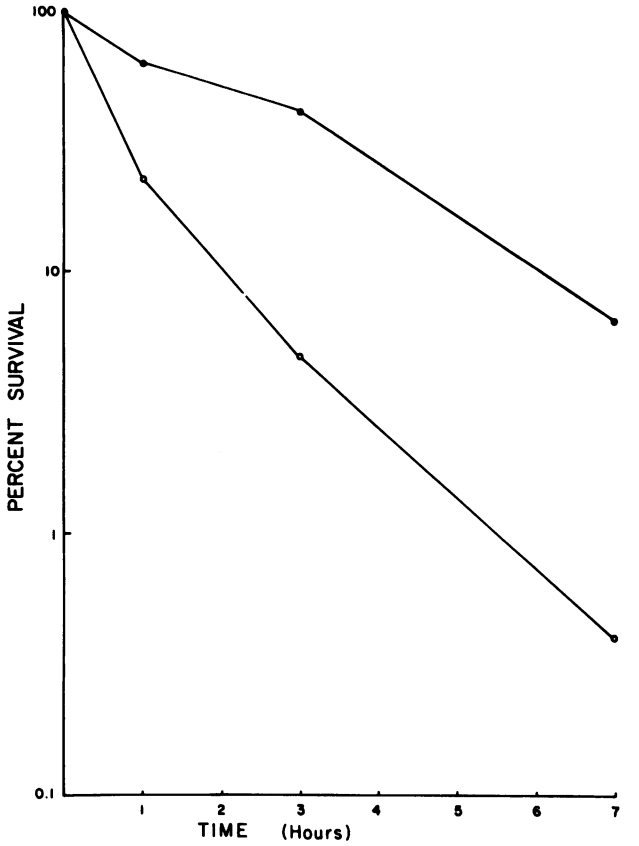


FIG. 3. Stability of wild-type PrV(Ka) and of a gIII$^-$ deletion mutant of PrV(Ka). Virus in Eagle medium (approximately $10^6$ PFU/ml) was incubated at 45°C for various periods and plaque assayed on MDBK cells to determine the residual titer. Symbols: ●, PrV(Ka); ○, gIII$^-$ mutant.

**Effect of mutations in gIII on syncytium formation and cell-to-cell spread.** No difference between the cytopathic effects of wild-type virus and gIII$^-$ mutants was observed. In cultures infected at high multiplicity, extensive syncytia were seen in RK cells infected with either virus. The sizes of the plaques, both on RK and MDBK cells, were also the same for wild-type virus and gIII$^-$ mutants at 3 or 4 days after inoculation. At low multiplicities of infection, gIII$^-$ mutants gave rise to localized plaques in MDBK cells incubated in liquid medium, whereas cultures similarly infected with the wild type showed generalized cytopathic effects much more rapidly. Furthermore, the spread of cytopathic effects observed in cultures infected at low multiplicity and incubated in liquid medium containing antiserum was affected to a much lesser extent in gIII$^-$ mutant-infected cells than in wild-type virus-infected cells. These observations are consistent with the view that although gIII is important in adsorption, cell-to-cell spread is not affected by its absence.

**Effect of mutations in gIII on virus release.** It has been suggested that gIII$^-$ mutants are not released as effectively

TABLE 5. Release of gI$^-$ and gIII$^-$ deletion mutants of PrV(Ka) from infected cells[a]

| Virus | Total no. of virions ($10^3$ cpm) (% extracellular)[b] | Specific infectivity[c] |
|---|---|---|
| Expt 1 | | |
| Wild type | 6,440 (64) | 1,600 |
| gIII$^-$ | 5,870 (58) | 330 |
| gI$^-$ | 6,230 (62) | 1,400 |
| gIII$^-$/gI$^-$ | 5,510 (45) | 110 |
| Expt 2 | | |
| Wild type | 21,800 (87) | ND[d] |
| gIII$^-$ | 13,210 (82) | ND |
| gI$^-$ | 21,000 (79) | ND |
| gIII$^-$/gI$^-$ | 17,880 (51) | ND |

[a] RK cells were treated with medium containing 5-fluorouracil (9) (to inhibit cellular DNA synthesis) and infected with the various virus mutants at a multiplicity of 4 PFU per cell. The cells were incubated at 37°C in medium containing [$^3$H]thymidine (50 μCi/ml) for 30 h (experiment 1) or 40 h (experiment 2). Extracellular virus was obtained from the culture fluid after its clarification by centrifugation at 5,000 × *g* for 5 min. Intracellular virus was obtained from the cells that were scraped from the surface of the plate and combined with the pellets obtained from the clarified culture fluid. Virus was purified as described in Materials and Methods, and the amount of radioactivity, as well as the amount of infectious virus, was determined.

[b] Counts per minute associated with the peaks of purified extracellular plus intracellular virions. Numbers in parentheses show the percentage of total virions that were extracellular.

[c] PFU/cpm in total virions (extracellular plus intracellular).

[d] ND, Not determined.

from the infected cells as are wild-type virions (11, 29). The experiments described below were performed to determine (i) the effect of defects in gIII$^-$ on virus release and (ii) whether gIII$^-$ mutant-infected cells (which produce a lower titer of infectious virus) produce the same number of viral particles as do wild-type-infected cells.

The total number of viral particles produced by cells infected with wild-type virus or with a gI$^-$, a gIII$^-$, or a gI$^-$/gIII$^-$ mutant was approximately the same (as estimated by the amount of [$^3$H]thymidine-labeled DNA associated with the purified virus peaks) (Table 5). The amount of virus that was released by 30 or 40 h after infection from cells infected with either the gIII$^-$ or the gI$^-$ deletion mutant was also not significantly smaller than that released from cells infected with the wild type. Thus, a defect in either gIII or gI alone did not detectably affect virus release under the experimental conditions used. Some effect on release, as a result of inactivation of gIII in the Becker strain of PrV, was observed at early stages of infection (Enquist, personal communication). It is possible that a defect in gIII in the PrV(Ka) strain also affects virus release but that this defect is detectable only at early stages of infection. Alternatively, the absence of gIII alone may affect virus release differently in different cell types.

Although no effect on the release of gIII$^-$ or gI$^-$ mutants was detected in our experiment, the release from the cells of the gI$^-$/gIII$^-$ double mutant was significantly less efficient. In experiment 1, of the total virions produced, more than 60% of the wild-type virus but only 45% of the gI$^-$/gIII$^-$ mutant was released from the cells; in experiment 2, of the total virions produced, more than 80% of the wild-type virus but only 51% of the gIII$^-$/gI$^-$ mutant was released from the cells. Thus, a defect in gIII, in conjunction with a defect in gI, significantly affected virus release. We have previously shown that gI affects virus release in conjunction with other (unknown) viral functions (19). The results presented here show that gIII is one of these functions.

TABLE 6. Effect of glycoprotein gIII$^{Ka}$ on the release of Bartha derivatives[a]

| Virus strain | Phenotype | Total virus ($10^3$ cpm) | % Released (extracellular) |
|---|---|---|---|
| Bartha | gIII$^B$ gI$^-$ gp63$^-$ | 4,260 | 2 |
| Bartha gIII$^{Ka}$ | gIII$^{Ka}$ gI$^-$ gp63$^-$ | 6,460 | 67 |
| Bartha 43/25a | gIII$^B$ gI$^+$ gp63$^+$ | 5,390 | 33 |
| Bartha 43/25agIII$^{Ka}$ | gIII$^{Ka}$ gI$^+$ gp63$^+$ | 6,890 | 71 |
| PrV(Ka) | | 6,990 | 69 |

[a] RK cells were infected with the appropriate virus (multiplicity of infection, 10 PFU per cell) and incubated in Eagle medium containing [$^3$H] thymidine (50 μCi/ml). At 24 h postinfection, extracellular and intracellular viruses were purified as described in Materials and Methods and Table 5, footnote *a*.

The specific infectivity of the gIII$^-$ mutants is, in this experiment, between 5 and 15 times lower than that of wild-type PrV or of a gI$^-$ mutant (Table 5), indicating that although gIII$^-$-infected cells produce as many virus particles as do wild-type-infected cells, the titer of the virus produced is lower.

**Release from RK cells of Bartha derivatives which have acquired a gIII gene from PrV(Ka).** To determine whether gIII mediates its effects on release in conjunction with gI only or whether it does so in conjunction with other viral functions as well, we determined whether replacement of the resident gIII gene of another virus strain (the Bartha strain, which is known to be released inefficiently from RK cells [3, 19]) by a gIII gene of PrV(Ka) would affect virus release. The gIII glycoprotein of the Bartha strain (gIII$^B$) behaves in an abnormal manner (1, 3). This is due to a modification of the Bartha gIII gene, as indicated by the fact that replacement of the gIII gene of the Becker strain with a Bartha gIII gene results in a virus variant which produces a Bartha-like gIII glycoprotein (Enquist, personal communication). The resident Bartha gIII gene (gIII$^B$) was therefore replaced by the gIII gene of PrV(Ka) (gIII$^{Ka}$) in the parental Bartha strain, as well as in the marker-rescued Bartha strain (43/25a), and the release of these Bartha variants from RK cells was ascertained (Table 6).

As previously reported (3, 19), the parental Bartha strain is released poorly from RK cells. Bartha 43/25a (which had acquired the genes encoding glycoproteins gI and gp63 as a result of marker rescue [15]) is released to a greater extent than is the parental Bartha strain, but it is not released as well as is wild-type PrV(Ka). After acquisition of the gIII$^{Ka}$ gene, the Bartha strain (which does not express gI or gp63) was released from RK cells more efficiently than Bartha 43/25a (which expresses gI and gp63) and as efficiently as was Bartha 43/25a gIII$^{Ka}$ (which expresses gI and gp63 and gIII$^{Ka}$) or wild-type virus. Thus, although glycoproteins gI and gp63 play a role in virus release and partly restore to the Bartha strain the ability to be released, gIII$^{Ka}$ alone was sufficient to completely restore to the Bartha strain the property of being released efficiently from RK cells.

These results show clearly that gIII is involved in virus release. Its effect is, however, detected to a significant extent in RK cells only when examined against certain genetic backgrounds of the viruses. Thus, although a dramatic effect on release was observed after acquisition by the Bartha strain of a gIII gene derived from PrV(Ka), an effect on virus release after inactivation of gIII in wild-type PrV(Ka) was not detectable in our experiments. Inactivation of gIII, however, did affect the release of a mutant of PrV(Ka) that was also defective in gI.

**Adsorption of Bartha derivatives.** The above results show that the gIII gene of the Bartha strain is defective and that this defect contributes to the poor release of that virus strain from RK cells. Since gIII also plays a role in virus adsorption, we ascertained whether there would be a difference in the adsorption rates of Bartha and Bartha gIII$^{Ka}$, as well as Bartha 43/25a and Bartha 43/25a gIII$^{Ka}$. To this end, an experiment similar to the one in Fig. 2 was performed with these virus variants.

No difference between the adsorption characteristics of PrV(Ka) or any of the Bartha variants was found (data not shown). Thus, although the Bartha gIII gene is functionally defective in release of the virus from the cells, adsorption of the Bartha strain to the cells occurs efficiently.

## DISCUSSION

Glycoprotein gIII of PrV, a homolog of gC of HSV, is nonessential for growth in cell culture (1, 28, 32). However, cells infected with gIII$^-$ virus do not appear to yield virus titers that are as high as those of cells infected with wild-type virus (11, 29). The experiments described in this report were designed to identify the reasons for this reduction in virus titer and to identify the role of gIII in the various interactions of the virus with its host cell.

We have reported previously (8) that monoclonal antibodies against gIII inhibit the stable adsorption of PrV to its host cells (i.e., the ability of the virus to remain cell associated after extensive washing of the monolayers). It was not clear, however, whether the inhibitory effect of these monoclonal antibodies was the result of a direct involvement of gIII in adsorption or whether it affected adsorption by steric hindrance only. We show here that mutants defective in gIII do not associate with the cells as readily as does wild-type virus and, furthermore, that in contrast to wild-type virus, gIII$^-$ mutants can be more easily dislodged from the cell surface by washing than wild-type PrV is.

It is interesting that although gIII appears to play an important role in adsorption, gIII$^-$ mutants are still able to penetrate the cell and initiate infection, but at a lower rate and efficiency. Thus, either there is an alternative, gIII-independent mechanism by which the virus can gain entry into the cells or glycoprotein gIII is only one of several viral surface proteins (or part of a complex of viral proteins) that enhances attachment and penetration of the virus into the cells.

The finding that a viral glycoprotein may facilitate adsorption but is not essential to the initiation of the infective process is not unique for PrV. Thus, glycoprotein G of vesicular stomatitis virus plays an important role in viral attachment to cells (4), but this virus may also initiate infection as a result of nonspecific binding to the cells (13). Furthermore, it has also been suggested that different vesicular stomatitis virus proteins (or determinants of the same proteins) may recognize different cell receptors (25, 30).

PrV that is complexed with monoclonal antibodies against gIII cannot initiate infection. It appears, therefore, that access of the virus to the cells, either by the rapid gIII-dependent mode or by the slower gIII-independent mode, is lost after treatment of the virus with anti-gIII monoclonal antibodies. It is possible that steric hindrance by the anti-gIII antibodies occurs in areas of the viral membrane in which the proteins (including gIII) that normally play a role in adsorption and penetration are present. Alternatively, binding of gIII, but not of most other viral glycoproteins, to the appropriate antibody may induce conformational changes in the viral membrane, thereby preventing initiation of infection. Since little is known concerning the roles different viral proteins may play in the interactions between the cellular receptors and viral attachment proteins, it may be premature to speculate about the mechanisms by which the interaction of the virus with a monoclonal antibody against gIII may interfere with both processes of virus adsorption.

Glycoprotein gIII is a homolog of gC of HSV, with which it shares sequence homology (27). However, although gIII of PrV appears to facilitate PrV adsorption, gC of HSV does not. Antibodies capable of neutralizing gC in the absence of complement have not been found, and even though gC has been shown to affect adsorption and penetration of HSV, the effects described have been negative. Thus, DeLuca et al. (5) have shown that gC$^-$ viruses enter cells at an increased rate compared with gC$^+$ viruses, and Epstein and Jacquemont (6) have shown that gC$^-$ HSV is able to penetrate some cell types that are resistant to wild-type gC$^+$ HSV. It appears, therefore, that despite the sequence homology between gIII of PrV and gC of HSV, these glycoproteins differ functionally in some respects.

The main conclusions that can be drawn from the results in this report are as follows. (i) gIII is a multifunctional glycoprotein: it plays an important role in virus adsorption; it affects virus stability; and it mediates the release of virus from the infected cells, particularly in conjunction with other viral functions (including glycoprotein gI). It is clear that the different effects of gIII are not different aspects of the same function. Deletion of gIII alone affects adsorption to the same extent as deletion of gIII and gI, but does not measurably affect virus release (at least under the experimental conditions we have used), whereas deletion of gIII and gI does. That gIII is a multifunctional protein can also be deduced from the results obtained with the Bartha strain. The resident gIII glycoprotein of the Bartha strain was found to be functionally defective in virus release but not in virus adsorption. It is clear, therefore, that these constitute two separate functions of gIII. (ii) The results presented in this report confirm and extend our previous conclusions (16, 19) that the effects of some viral functions are easily detectable only against the background of defects in other viral functions. We show here that gIII affects virus release in conjunction with defects in other functions, one of which is gI. (iii) Not unexpectedly, the initial interactions of PrV with its host cells are complex. gIII plays a role in virus adsorption; in its absence, the rate of virus adsorption is slowed significantly. However, gIII$^-$ virus is infectious, and therefore adsorption in the absence of gIII can occur by a mechanism that is not dependent on this glycoprotein.

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